Thrombin generation in plasma

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Thrombin Generation in Plasma: Its Assessment Via the Endogenous Thrombin Potential

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Introduction

Thrombin is a pivotal player in the intricate scenario that ensures haemostasis when triggered by injury but that leads to thrombosis when set off inappropriately. Thrombin generation is due to a complex enzymatic mechanism by which, in triggered plasma, thrombin is first formed and subsequently inactivated. *In vivo*, this process is closely intertwined with functions of blood cells and vessels. Nevertheless, all circumstances that reduce the thrombin generating capacity of plasma (e.g. every form of anticoagulation) have an antithrombotic and haemorrhagic effect, and all conditions that increase thrombin generation (e.g. deficiencies of antithrombin or the proteins C or S, APC resistance) foster thrombosis (see also Fig. 1). Therefore the thrombin generating capacity of plasma is one of the main determinants of haemostasis and thrombosis. In the management of anticoagulant therapy and for the detection of thrombosis and bleeding risks, we need a laboratory parameter that expresses this function. Since more than a century clotting times are used for this purpose, but they are insensitive to hypercoagulation and barely sensitive to moderate coagulation defects. Moreover a plasma sample clots already when only 10–20 nM of thrombin are formed, at a moment that the large majority (>95%) of thrombin has still to be generated. Consequently, features of the thrombin generating process that take place after clotting has occurred are not reflected in the clotting time. A classical example is the thromboplastin time, that is hardly prolonged even if the amount of thrombin formed is substantially decreased by the presence of heparin (see Fig. 2). There is a need for a parameter that decreases upon hypocoagulation of any kind and increases in hypercoagulability, in proportion to the amount of active thrombin that is formed during coagulation in a plasma sample. The limited data available at this moment suggest that the ETP, contrary to the PT and the APTT, is a parameter that decreases upon hypocoagulation of any kind and increases in hypercoagulability, in proportion to the thrombin forming capacity of a plasma sample (1,2).

The potency of thrombin generation of the plasma is not to be confused with the extent of ongoing thrombin generation in the body. Fragment 1.2 e.g. reflects that thrombin is being generated in the body. It is a smoke detector that reports an ongoing fire. A test for hypo- or hypercoagulability however should indicate the fire risk, i.e. the potential thrombin-forming capacity of the non triggered system. The difference between the two appears e.g. in DIC (disseminated intravascular coagulation) with consumption coagulopathy, where the indicators of ongoing coagulation are high but the capacity of the plasma to generate thrombin is low.

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The mechanism of thrombin generation

In the mechanism of thrombin generation three essentially different types of action can be recognized, that can be represented by three axis: X) Thrombin production and inactivation in the strict sense of the word, Y) Modulation of the reaction velocity and Z) Localization at the site of vascular damage. In Fig.1 the X and Y axis are shown, the Z axis is not rendered. These processes cooperate at the level of single enzyme complexes; e.g. prothrombin conversion as such is carried out by factor Xa, with a velocity that is determined by the availability of factor Va and the process remains confined to the surface of a procoagulant membrane.

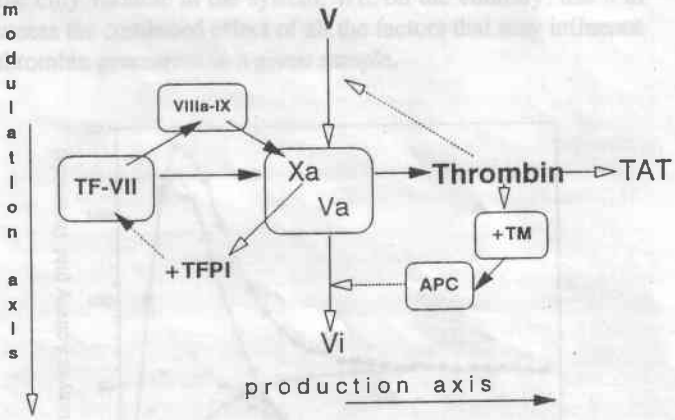

Fig. 1. A scheme of thrombin generation. Lines with open arrow heads: chemical conversion; lines with black arrow heads: activation of a proenzyme (e.g. Xa Æ Thrombin= factor Xa converts prothrombin into thrombin); Dotted lines with open arrow heads: activating action; Dotted lines with black arrow heads: inhibitory action.

The ordinate. The core of the thrombin generation mechanism is the production inactivation axis. Thrombin is produced by the well known "cascade" mechanism, i.e. the tissue factor factor VIIa complex (TF-VIIa) activates factor X, factor Xa activates prothrombin (3–5). The resulting thrombin is inactivated by antithrombin (AT) (~77%), α_2 macroglobulin (α_2M)(~14%) and minor inhibitors (~9%). The production is limited in time by the tissue factor pathway inhibitor (TFPI): When a sufficient amount

of factor Xa is formed, the generation of more factor Xa is shut down because a XaTFPI complex forms that efficiently inhibits further activity of TFVIIa (6). Factor IX is activated by the factor VIIaTF complex like factor X is. Together with factor VIIIa it forms an alternative factor X activator that is not inhibited by TFPI but the activity of which is modulated by factor VIII activation and inactivation (see below). This reinforcement loop (the Josso loop) probably constitutes an escape mechanism that, at low TF, prevents precocious arrest of factor Xa formation via the TFPI mechanism.

The abscissa. The second axis is modulation of thrombin generation velocity via activation and inactivation of factor V and factor VIII. In their activated form these factors enhance the proteolytic activity of their partners (F.Xa and F.IXa resp.) approximately a thousand fold (3–5). The appearance and disappearance of factor Va (VIIIa) is governed by thrombin. Activation of factor V in plasma can be inhibited by hirudin, so factor Xa is not likely to be the physiological activator (1). The inactivation of factor Va (VIIIa) is also thrombin dependent because the scavenger of factor Va (VIIIa), activated protein C, is generated by the thrombomodulin thrombin complex (7,8).

Factor V is probably preferably activated by meizothrombin at a phospholipid surface (9) whereas factor VIII is kept in solution by von Willebrand factor and activated by thrombin in free solution. Meizothrombin is insensitive to the AT enhancing effect of heparin (10), which may explain why the thromboplastin time is relatively insensitive to heparin. Under the conditions of the APTT, however, the lag time of thrombin formation is mainly dependent upon the feedback activation of factor VIII by free thrombin, and therefore is sensitive to heparin.

The third axis. Several reactions in thrombin generation (indicated by boxes in Fig. 1) proceed to a significant extent only when the reactants are adsorbed onto a phospholipid membrane that contains phosphatidylserine (PS). Such membranes occur at sites where endothelial integrity is lost, so that thrombin generation is confined to lesions in the vasculature. PS is almost exclusively found at the cytoplasmatic side of cell membranes and is hardly exposed at the exterior of the cell (11). When a vessel is damaged, cell damage will offer some PS containing membranes to the plasma. TF meets factor VII and so starts the coagulation cascade. Moreover collagen is exposed to which can further cause platelet adhesion (12). As soon as traces of thrombin are formed, the simultaneous action of thrombin and collagen induces transbilayer movement of PS in the platelet membrane and the platelet surface becomes procoagulant (11).

PS containing membranes enhance the velocity of thrombin generation because clotting factors meet more easily when adsorbed at these surfaces. Prothrombin, e.g., reaches the prothrombinase complex by diffusion in the plane of the surface (13). The apparent Km of prothrombin conversion, (i.e. the concentration of prothrombin in solution that is necessary to half saturate prothrombinase) decreases proportionally with the size of the procoagulant surface that is available around a prothrombinase molecule. Thrombin formation by a prothrombinase complex on a large procoagulant surface is so fast that, under physiological conditions, the velocity of its action is determined by the velocity at which prothrombin can be delivered to the surface rather than by the capacity of the enzyme (k_{cat}= 3600 min^{-1}, Km= 5 nM, 13).

The thrombin generation mechanism is so replete with positive and negative feedback mechanisms that its kinetics show a strongly nonlinear behaviour. On the one hand parameters may vary without changing the output very much, on the other hand, small changes in one or more of the constituent activities may have unexpected, and unexpectedly large, effects on the output (14,6). This precludes that any of the isolated functions within the mechanism will adequately render the function of the whole. In order to assess the function of the thrombin generation mechanism one therefore has to measure thrombin, under conditions that are as near as possible to those *in vivo*.

The endogenous thrombin potential

We can imagine that in an area where thrombosis develops, the number of natural substrate molecules (fibrinogen, membrane receptors etc) that is cleaved by thrombin, determines the extent of the thrombotic response. If the thrombin substrate is not exhausted (which fibrinogen e.g. easily is) then the number of molecules converted by thrombin is proportional to both the concentration of this enzyme and the time that it can act, i.e. to the area under the thrombin time curve (Fig.2). Therefore this area reflects the amount of (natural) substrate that could potentially be converted by the thrombin generated in the sample, also under *in vivo* conditions. We called it the endogenous thrombin potential (ETP) (2). As such it is not new. The area under a thrombin generation curve has been used by Biggs and Macfarlane (15) to determine prothrombin, making the prothrombin concentration the only variable in the system. We, on the contrary, use it to assess the combined effect of all the factors that may influence thrombin generation in a given sample.

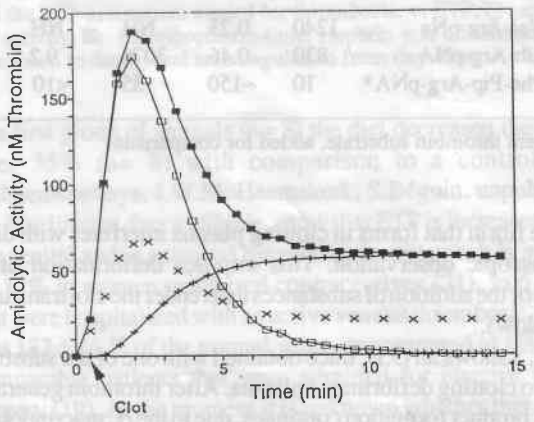

Fig. 2. A thrombin generation curve obtained by subsampling. ■ : amidolytic activity on S2238; □ : thrombin activity; + : α_2M-IIa activity; x: amidolytic activity in the presence of 0.05 U/ml of unfractionated heparin.

After thrombin generation is over and free thrombin has disappeared we measure a residual amidolytic activity (Fig.2). This is due to the α_2 macroglobulin thrombin complex (α_2M-IIa). A simple mathematical procedure allows to split the experimental curve into the part due to free thrombin and

the part that is due to this complex (Fig.2, see also below).

How to measure the endogenous thrombin potential

The obvious way to determine the ETP is to calculate the area under the thrombin time curve such as it can be obtained by subsampling from a clotting plasma sample (Fig.2). This method is labour intensive and unsuitable for use in the clinical laboratory. Another approach is to simply add an artificial thrombin substrate (e.g. a chromogenic substrate) to the sample and determine the amount of product that is converted after the thrombin generation process is over. If the substrate is not exhausted, then the concentration of product will be proportional to the amount of any other (physiological) substrate that could potentially be converted by the thrombin generated in that plasma, i.e. to the ETP. There are some practical problems though. In the first place none of the conventional thrombin substrates can be used because any concentration that can be added will be consumed before thrombin generation is over. Thus we need a substrate that is cleaved slowly. Yet it has to be specific for thrombin, because, if it would bind significantly to another clotting enzyme, it would interfere with the thrombin generation process. Preferably the substrate should be consumed to a limited extent (<10%), in order for the product formed to remain linearly proportional to the surface under the thrombin generation curve. We found two substrates that fulfil these conditions (Table 1).

Table 1. Properties of two chromogenic substrates suitable for measuring the ETP.

Substrate	Thrombin		Factor Xa	
	Km (mM)	kcat (s-1)	Km (mM)	kcat (s-1)
Msc-Val-Arg-pNa	1240	0.23	NH	NH
MZ-Aib-Arg-pNA	830	0.46	3070	9.2
H.D-Phe-Pip-Arg-pNA*	10	~150	35	<10

*A current thrombin substrate, added for comparison.

The fibrin that forms in clotting plasma interferes with direct spectroscopic observation. This imposes defibrination of the sample or the addition of substances that render the clot translucent (see below).

Fig. 3 shows an O.D. trace obtained with one of the substrates added to clotting defibrinated plasma. After thrombin generation is over, product formation continues, due to the α_2 macroglobulin thrombin complex (α_2M-IIa). Via a similar mathematical procedure as used to obtain the thrombin curve in Fig.2, the O.D.trace can be dissected into the part that is due to free thrombin and the part that is the result of the action of the α_2M-IIa complex on the substrate (Fig. 3).

The O.D. signal [P(t)] consists of two parts. One [A(t)] is generated by the action of free thrombin on the added substrate and the other [B(t)] by α_2M-IIa action. α_2M-IIa is formed with a velocity proportional to the concentration of free thrombin. The amount of α_2M-IIa present at any

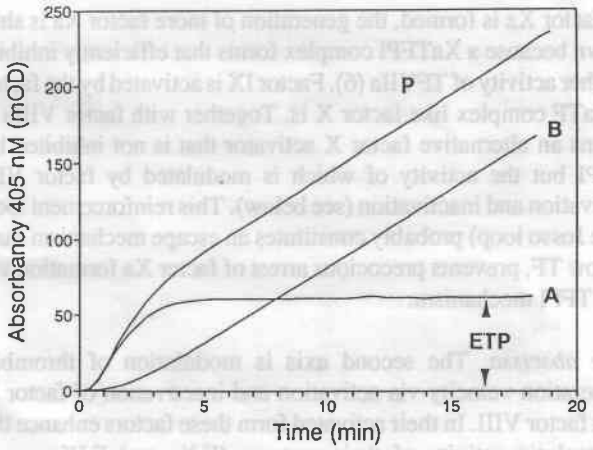

Fig. 3. Continuous registration of the conversion of a slow thrombin substrate during coagulation of plasma. P: Experimental curve; A: Substrate conversion due to thrombin activity; B: substrate conversion due to α_2M-IIa activity. A and B are calculated from P as indicated in the text.

moment therefore is proportional (constant = k) with the time integral of free thrombin. So B(t) is the integral of A(t) multiplied by k. In practice the integral can be replaced by the sum of all values of A at that moment available. It follows that at measuring point n in time, the total amount of product formed (Pn) equals the amount converted by free thrombin (A_n) plus the amount of product formed by $k.(A_0+A_1+.....+A_{n-1})$, so $P_n = A_n + k\ (A_0+A_1+.....+A_{n-1})$. It follows that A_n equals $(1-k).A_{n-1}+(P_n-P_{n-1})$. In this way the course of An can be calculated from the experimental data (i.e. the course of P_n) if k is known. This (sample dependent) constant can be found by trial and error, because, from the moment on that no free thrombin is left, A_n will not increase further. It is easy to write a program in which a time series of P is entered and that calculates a time series of A with any assumed value of k. That value of k that renders a steady end level of A in the end phase of the reaction, when no more free thrombin is generated, is the right value. The steady end level of A then is proportional to the thrombin potential by a factor $F[=(Km+S)/k_{cat}.S]$, with S being the substrate concentration and Km and k_{cat} the kinetic constants of the conversion of the substrate by thrombin.

The thrombin dependent part of the curve can also be obtained directly, after inactivation of α_2-M, either enzymatically or by reacting its internal S-cysteinyl-g-glutamyl thiolester. Hydroxylamine, at 100 mM, effectively destroys $\alpha_2$macroglobulin activity. As an additional benefit it renders the fibrin clot translucent, probably because it constitutes a substrate for factor XIIIa that competes with fibrin and hence prevents the light scattering that results from crosslinking. We still have to determine if, and to what extent, hydroxylamine interferes with the normal thrombin generation mechanism. Preliminary results lead us to suppose that by addition of hydroxylamine the α_2M effect can be abolished, so that the experimental O.D. trace takes the form of curve A in Fig. 3 and the ETP can be determined as a simple endpoint measurement.

We adapted the ETP assay in defibrinated plasma to a Cobas laboratory automaton, which enables us to carry out more than 50 determinations per hour. The ETP in platelet rich plasma is not readily automated. In such plasma defibrination is impossible without removing the platelets and the addition of hydroxylamine may damage the platelet membrane. For the time being the ETP of a platelet rich sample has to be determined via a

thrombin generation curve obtained by subsampling.

The ETP of normal individuals equals 382 nM.min ± 52 (S.D.,n=86) (As the ETP represents a surface on the concentration time plane, its dimensions are concentration multiplied by time, i.e. nM.min. The value may vary somewhat between labs, dependent upon the calibration factor used to convert OD changes into concentrations of thrombin. In clinical practice it is convenient to express the ETP as a % of the normal value). The ETP is hardly dependent upon the concentration of thromboplastin used. We prefer to measure the ETP after triggering with dilute recombinant human tissue factor because we think this to be nearest to the physiological situation. Triggering the ETP via the contact activation mechanism is equally well possible (Fig. 4).

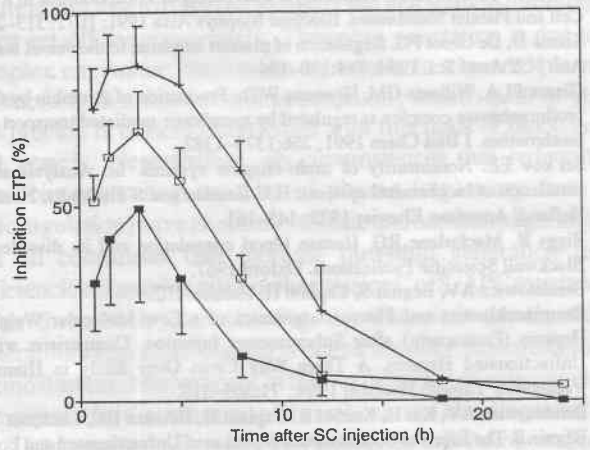

Fig. 4. The course of the ETP after subcutaneous injection of unfractionated and low molecular weight heparin. In 12 healthy volunteers heparin injected subcutaneously at t = 0. The mean and SEM are given of the activity of the ETP compared to the preinjection sample.
■ : 5000 IU unfractionated heparin; □: 40 mg enoxaparin; + : 75 mg enoxaparin.

The ETP in hypo- and hypercoagulation

Up to this moment there are a number of different observations that suggest the ETP to be a sensitive indicator of hyper- and hypocoagulability but validation studies are still to be performed. The ETP is more sensitive to the effect of both unfractionated- and low molecular weight heparin than the APTT. A dose dependent effect on the ETP can be observed until 12–18 hours after injection of LMW Heparin (Fig. 4) (16). The ETP in platelet rich plasma is much more affected by low molecular weight heparins than by unfractionated heparin (17). In oral anticoagulation the ETP is dependent upon variations in the concentration of each of the vitamin K dependent clotting factors (Fig. 5) (18). Half normal ETP values are observed at about the following clotting factor concentrations: Prothrombin: 50%, FVII: 2%; FX: 10%. It is interesting to note that, when switching from heparin therapy to oral anticoagulation, the ETP, unlike PT, APTT and anti-factor Xa activity seems to render the level of the mixed treatment (Fig. 6) (19). To our surprise the ETP, when measured in whole blood, decreased under the influence of aspirin (20). In an ongoing study, rats are being tested for the influence of a fish oil containing diet on the ETP. It was found that

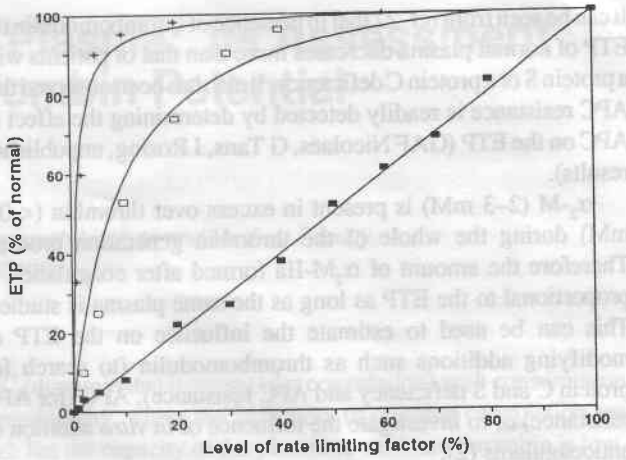

Fig. 5. The influence of the level of vitamin K dependent factors on the ETP. The factors II, X and VII were independently varied between 0 and 100% and the influence on the ETP was measured. ■: prothrombin; □ : factor X; +: factor VII.

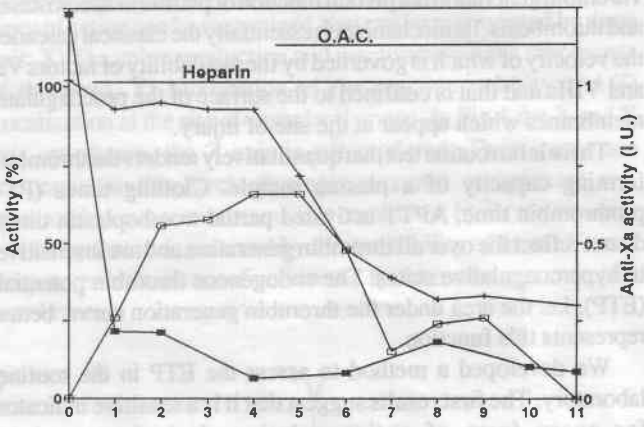

Fig. 6. The course of thromboplastin-time, anti-factor Xa activity and the ETP in a patient treated for thrombosis. +: ETP; □ : anti-factor Xa activity; ■: thromboplastin-time. Heparin was administered from admission to day 9, oral anticoagulation from day 4 on.

in a first group of animals (n= 8) the diet decreased the ETP by over 35% (n= 8) with comparison to a control group (C.Nieuwenhuys, J.W.M. Heemskerk, S.Béguin, unpublished).

The limited data available, show that ETP is increased both in congenital and in acquired hypercoagulant states. It is increased by 12% in women using oral contraceptives (21). In 6 patients that were hospitalized with an active venous thrombosis, the ETP was 132 ± 16 % of the normal value, as compared to 109 ± 13 in comparable patients that were admitted for elective vascular surgery (19). At the moment it is unknown whether this is due to the active process or to a pre-existing thrombotic tendency. At present we are investigating the coagulation status of young stroke patients. From the first 7 patients, 6 have an ETP in PRP (platelet rich plasma) that is significantly higher than a sex and age matched normal control, and the mean is 23 ± 8 % higher than the mean of the controls. The ETP in PPP (platelet poor plasma) was not systematically increased (16 ± 14%), yet in two out of 7 patients it was increased by 33% and 40% (K. Faber, S.Béguin, preliminary results).

We found the ETP to be increased in antithrombin deficiency.

It can be seen from ref. 22 that in presence of thrombomodulin the ETP of normal plasma decreases more than that of patients with a protein S or a protein C deficiency. It also has been observed that APC resistance is readily detected by determining the effect of APC on the ETP (GAF Nicolaes, G Tans, J Rosing, unpublished results).

α_2-M (2–3 mM) is present in excess over thrombin (< 0.3 mM) during the whole of the thrombin generation process. Therefore the amount of α_2M-IIa formed after coagulation is proportional to the ETP as long as the same plasma is studied. This can be used to estimate the influence on the ETP of modifying additions such as thrombomodulin (to search for protein C and S deficiency and APC resistance), APC (for APC resistance) or to investigate the influence of *in vitro* addition of anticoagulants (2).

Summary

Thrombin generation is a pivotal function of plasma in haemostasis and thrombosis. Its mechanism is essentially the classical cascade, the velocity of which is governed by the availability of factors Va and VIIIa and that is confined to the surface of the procoagulant membranes which appear at the site of injury.

There is no routine test that quantitatively renders the thrombin forming capacity of a plasma sample. Clotting times (PT-prothrombin time, APTT-activated partial tromboplastin time) do not reflect the over all thrombin generation and are insensitive to hypercoagulative states. The endogenous thrombin potential (ETP), i.e. the area under the thrombin generation curve, better represents this function.

We developed a method to assess the ETP in the routine laboratory. The first results suggest that it is a sensitive indicator for every form of anticoagulation. It is increased in hypercoagulable states thus far studied, both congenital and acquired and can be designed to indicate deficiencies in protein C and S and APC (activated protein C) resistance.

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